Volume 336, number 3, 403–407 FEBS 13450 December 1993

Induction of cytoplasmic factors that bind to the 3′ AU-rich region of human interferon β mRNA during early development of *Xenopus laevis*

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Received 10 October 1993; revised version received 16 November 1993

Certain endogenous *Xenopus* mRNAs, carrying a destabilizing 3′ AU-rich sequence, are unusually very stable in oocytes and become unstable only after fertilization. In addition, heterologous short lived mRNA, containing 3′ AU-rich sequences, appear to be very stable when injected into *Xenopus* oocytes. In the present study, a human interferon β (hu-IFNβ) mRNA, carrying the destabilizing 3′ AU-rich element, was used as a probe to identify *Xenopus* proteins that specifically bind to the 3′ AU-rich element as well as to study their relative levels during early embryonic development. While three major proteins that specifically bind to the 3′ AU-rich element were detected in human SV80 cells, that naturally express hu-IFNβ (proteins termed AU-F1, F2 and F3), only two proteins, migrating similarly to the SV80 AU-F1 and AU-F3, were detected in cytoplasmic extracts from *Xenopus* oocytes or eggs. Following fertilization, the intensity of the *Xenopus* AU-F1 and AU-F3 proteins increased considerably and a new protein, corresponding to SV80 AU-F2, was also detected. Cyclohexamide applied either at the morula or at the early blastula stages reduced the intensity of the AU-binding factors, while actinomycin D did not, indicating that the levels of these factors during these stages are regulated posttranscriptionally. In contrast, application of each of these metabolic inhibitors at the late blastula stage increased the intensity of the AU-binding proteins. The possible function of these AU-binding factors in regulating the expression and half life of AU-rich mRNAs is discussed.

Human interferon β; AU-rich region; RNA-binding proteins; *Xenopus laevis*; Early embryogenesis; mRNA stability

1. INTRODUCTION

Many short lived mRNAs, including c-*myc*, c-*fos* and hu-IFNβ, share a common AU-rich sequence at their 3′ untranslated regions (UTR), which confer cytoplasmic instability [1–3]. Interestingly, during the reproductive stage of *Xenopus*, these mRNAs appear to accumulate in oocytes and eggs and decreases substantially after fertilization [4–7]. Even heterologous mRNAs containing the destabilizing 3′ AU-rich element, like hu-IFNβ, that turnover rapidly in their natural cells, are very stable upon injection into *Xenopus* oocytes. [8]. It was also suggested that *Xenopus* oocytes may lack some *trans*-acting factors that are necessary for the rapid turnover of these mRNAs [2].

Recent studies have identified several cytoplasmic proteins from a variety of eukaryotic cells that specifically bind the 3′ AU-rich sequences of various mRNAs and may play a role in their translation and instability [9–13]. In this study, we used hu-IFNβ mRNA as a probe to identify 3′ AU-binding activity in *Xenopus* oocytes and eggs as well as during the early embryonic development. Two specific AU-binding proteins were detected in oocytes and eggs, and an additional protein was detected only after fertilization.

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2. MATERIALS AND METHODS

2.1. *Plasmids and enzymes*

The hu-IFNβ constructs either carrying (pβA110) or lacking (pβAD79A110) the AU-rich sequence as well as the constructs 3′βA110 and 3′βD79A110 (Fig. 1A), used for studying the AU-binding activities, have been described previously [14]. Restriction endonucleases and T3 and T7 RNA polymerases were purchased from Boehringer Mannheim.

2.2. *Cell growth and preparation of cytoplasmic extracts*

Mature females of *Xenopus* laevis were injected into their dorsal lymphatic bags with 100 units of human chorionic gonadotropin (Pregnyl, Organon Holland) and 6 h later with a further 600 units. Ovulation of mature jellied eggs was induced 10–12 h after the second injection. Eggs were squeezed from the females onto 1 × MMR solution (0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES pH 7.8 and 0.1 mM EDTA) and fertilized by addition of sperm from macerated testes. Fertilized eggs were then dejellied by gentle swirling (5 min) in 2% cysteine freshly prepared in 0.1 × MMR solution, pH 7.8, followed by several washing with 0.1 × MMR. Embryos were then allowed to develop in 0.1 × MMR at room temperature and staged as described by Nieuwkoop and Faber [15]. Samples were removed at various times during the early embryonic development, frozen by liquid nitrogen and stored at −80°C until used. Oocytes and embryos were homogenized in a solution containing 25 mM Tris-HCl (pH 7.8), 0.1 mM PMSF, 0.5 mM leupeptin and 0.5% NP-40, by pipetting up and down and centrifuged (14,000 × *g*, 4°C, 10 min). The supernatant was collected and stored at −80°C. Embryos at various stages of development were also incubated for one hour in 0.1 × MMR solution containing 200 μg/ml cyclohexamide [16], or 25 μg/ml actinomycin D. The embryos were then washed several times and allowed to develop up to the late gastrula stage.

Human fibroblast SV80 cells [17], kindly provided by Dr. J. Chebath (Dept. Molecular Genetics and Virology, The Weizmann Insti-

tute of Science, Rehovot, Israel), were grown to 80% confluence in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum and 5% newborn calf serum at 37°C, 5–8% CO_2. The cells were collected and washed twice in phosphate-buffered saline and lysed by freeze-thawing in 25 mM Tris-HCl (pH 7.5) and 0.5% Nonidet P40. The homogenate was centrifuged at 14,000 × *g* for 10 min at 4°C, and the supernatant was collected and stored at −80°C until used.

2.3. *Analysis of mRNA stability in the oocytes*

In vitro transcription, injection of mRNAs into the oocytes, and maintenance of oocytes were as described [14, 18]. Following microinjection of approximately 2–5 ng of mRNA per oocyte, the oocytes were incubated in OR-2 medium [19] and sampled at various times after injection. Determination of RNA stability by RNase protection assay was performed essentially as previously described [20]. Ten μg of glycogen (Boehringer Mannheim) was used as a carrier instead of tRNA.

2.4. *UV cross-linking and label transfer*

Transfer of radioactivity from RNA probes to RNA-binding proteins was carried out as described previously [14]. The intensity of bands was analyzed by densitometry (Molecular Dynamics, Imagequant, USA).

3. RESULTS

In the present study, we used hu-IFNβ mRNA as a probe to identify specific proteins that bind to the 3′ AU-rich region and study their expression during oogenesis and early embryonic development of *Xenopus*. We first wished to test whether hu-IFNβ will be stable in *Xenopus* oocytes, similarly to the endogenous *Xenopus* mRNAs containing the 3′ AU-rich region

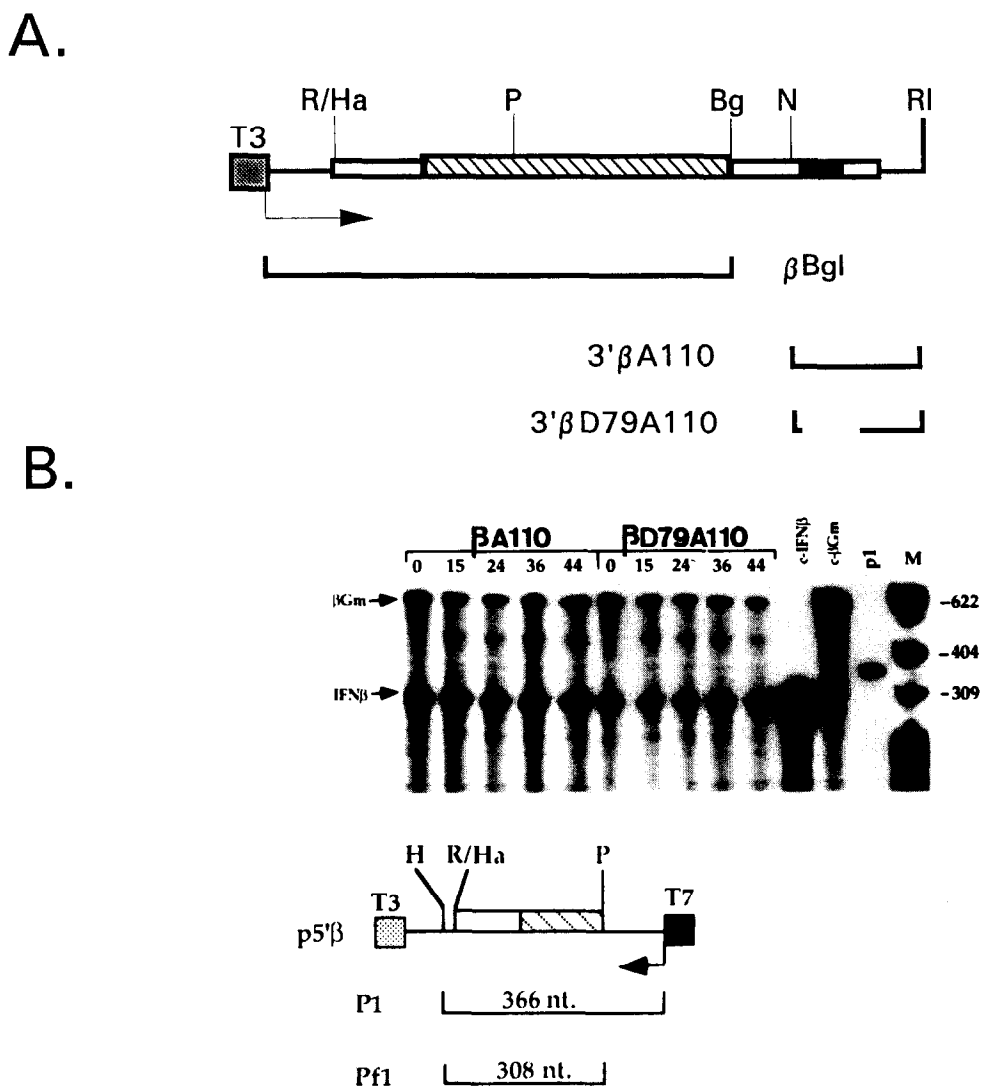

Fig. 1. (A) Schematic representation of hu-IFNβ constructs employed in the present study. The RNA probes βBgl, 3′βA110 and 3′D79A110, used to study AU-binding activity are indicated on the bottom. Symbols: ▨ coding region; □ 3′ UTR; ■ 3′ AU-rich sequence; Restriction enzymes: R = *Eco*RV, Ha = *Hae*III, P = *Pst*I, Bg = *Bgl*II, N = *Nde*I, H = *Hin*dIII. (B) The stability of βA110 and βD79A110 mRNAs in *Xenopus* oocytes. Stability was analyzed by RNase protection at various times after injection as indicated on the top of the figure. Human β-globin mRNA was injected with each mRNA as a reference. The specific protected fragments for hu-IFNβ and for β-globin (βGm) mRNAs are indicated by arrows at left. c-IFNβ and c-βGm are the protected fragments of noninjected βA110 and β-globin transcripts. The plasmid p5′β used for the protection assay of IFNβ transcripts is presented below Fig. 1B. P1 indicates the ^{32}P-labeled probe complementary to the 5′ end of hu-IFNβ mRNA, while Pf1 indicates the expected protected fragment. M indicates size markers of ^{32}P-labeled *Msp*I fragments of pBR322 with sizes in bp indicated at right.

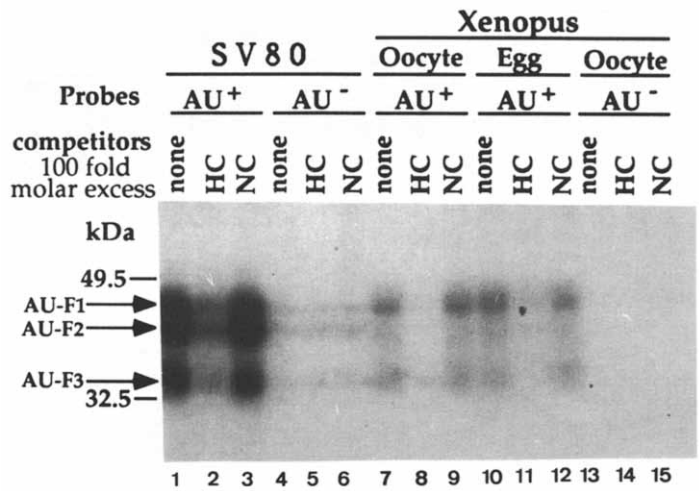

Fig. 2. AU-binding activity in cytoplasmic extracts of human fibroblast SV80 cells and in *Xenopus* oocytes and eggs. Fifteen μg of total cytoplasmic proteins were mixed with 10 femtomoles of either AU^+ (3′βA110) or AU^- (3′D79A110) RNA probes. After UV-cross linking and RNase treatment, the samples were subjected to electrophoresis on 10% polyacrylamide gel containing 0.1% SDS. Proteins were detected by autoradiography. HC indicates 100-fold molar excess of the non radioactive homologous competitor, while NC indicates 100-fold molar excess of the non radioactive non homologous competitor, βBgl RNA. Specific complexes are indicated by arrows. The position of molecular weight markers is indicated at left.

[4,5,6,7]. Two hybrid hu-IFNβ mRNAs, carrying the AU-rich region (βA110) or lacking it (βD79A110) were transcribed in vitro, and each of them was coinjected into oocytes with an equal amount of globin mRNA as a reference. The stability of both mRNAs was assayed at various times after injection by RNase protection, using an antisense RNA probe corresponding to the 5′ end of hu-IFNβ mRNA (illustrated at the bottom of Fig. 1B). Both mRNAs were equally stable in the oocytes for at least 44 h after injection (Fig. 1B).

Since the 3′ AU-rich sequences of hu-IFNβ mRNA and of other short-lived messages have been shown to confer mRNA instability in the natural systems, we hypothesized that the unusual stability of such mRNAs in *Xenopus* oocytes may be due to low levels or absence of *trans*-acting factors which specifically bind to this region and are involved directly or indirectly in mRNA decay. Such factors should exist in mammalian cells and may be induced during early embryonic development of *Xenopus*.

To test this hypothesis, we performed RNA binding assays by using the label transfer procedure [21]. The RNA probes, AU^+ (3′βA110) and AU^- (3′D79A110), used for these experiments and the competitor RNA (βBgl) are shown schematically in Fig. 1A. The binding activity of the 3′AU-rich sequence was first tested in a cytoplasmic extract from oocytes and eggs. For comparison, we used cytoplasmic extracts from SV80 human fibroblast cells that naturally express IFNβ and were previously shown to contain three AU-binding factors [14]. In order to select specifically for binding to the AU-rich sequence, the RNA was labeled with [α-^{32}P]UTP. Fig. 2, lane 1 shows that the AU^+ probe (3′βA110) transferred its radioactivity to proteins from SV80 cells to form three specific complexes (designated AU-F1, AU-F2 and AU-F3, see arrows) that migrated between 33 to 46 kDa (see also [14]). Only very faint bands could be detected on the autoradiogram when the SV80 extract was incubated with the 3′D79A110 AU^- probe (Fig. 2, lanes 4–6), implying that the proteins bound specifically to the AU-rich sequence. When the AU^+ RNA probe was incubated with cytoplasmic extracts from either *Xenopus* oocytes or eggs (Fig. 2, lanes 7 and 10) only two specific complexes were detected that migrated similarly to the SV80 AU-F1 and AU-F3. However, the intensity of these bands was much lower than their corresponding bands from the SV80 cells. Again, no complexes could be detected when the oocyte extracts were incubated with AU^- RNA probe (lanes 13–15). The specificity of the protein–RNA interactions was also assessed by competition experiments (Fig. 2). One hundred-fold molar excess of unlabeled homologous RNA (HC, AU^+ RNA), strongly reduced AU-binding activity, while a 100-fold molar excess of unlabeled non homologous RNA derived from βBgl (NC, AU^- RNA) did not (Fig. 2, cf. lanes 2, 8 and 11 with lanes 3, 9 and 12, respectively). No complexes could be detected when the cytoplasmic extracts were preincubated with proteinase K (data not shown), indicating that binding activity involved protein factors.

Next, we tested the developmental regulation of the AU-specific binding activity during early embryonic development of *Xenopus*. Following fertilization, the intensity of AU-F1 and AU-F3 increased until late gastrula, which was the latest time tested (Fig. 3, cf. lanes 1–3 with lanes 4–10). Densitometer tracing analysis estimated that the intensity of these proteins was increased by about 2.5-fold (data not shown). In addition, a new

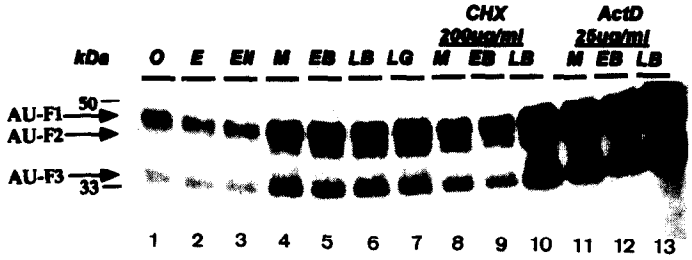

Fig. 3. The AU-binding activity during early embryonic development of *Xenopus*. Fertilized eggs were allowed to develop in MMR medium and groups of 20 embryos were harvested at the indicated developmental stages. 15 μg of total cytoplasmic proteins were mixed with 10 femtomoles of the AU$^+$ probe (3′βA110), and after UV-cross linking and RNase treatment, the samples were subjected to electrophoresis on 10% polyacrylamide gel containing 0.1% SDS. Proteins were detected by autoradiography. Specific complexes are indicated by arrows, while the position of molecular weight markers is indicated on the left. Lanes 1–7 represent the respective AU-binding proteins from oocytes (O); one cell embryos (E); four cell embryos (EII); morula (M); early blastula (EB); late blastula (LB); and late gastrula (LG). Group of 20 embryos were also pretreated for 1 h with cyclohexamide (CHX) (lanes 8–10) or with actinomycin D (ActD) (lanes 11–13) and allowed to develop to the late gastrula stage. Pretreatments were performed at the morula (lanes 8,11); early blastula (lanes 9,12) and late blastula (lanes 10,13) stages.

protein band, that migrated very similarly to the human AU-F2 protein, was detected after fertilization (Fig. 3, lanes 4–13). As the first 12 cell divisions following fertilization take place without transcription, the above results suggested that during this period, the increased levels of the AU-binding proteins was apparently regulated posttranscriptionally. To address this, we tested the effect of the metabolic inhibitors cyclohexamide and actinomycin D on the expression of the AU-binding proteins. Embryos were pretreated for one hour with cyclohexamide or actinomycin D at the morula, early blastula or late blastula stages, and then allowed to develop into late gastrula and harvested. The level of all three proteins at late gastrula was slightly decreased when embryos were pretreated at the morula or early blastula with cyclohexamide, but not with actinomycin D (Fig. 3, cf. lanes 8, 9 and 11, 12). Interestingly, as shown in Fig. 3 lanes 10 and 13, treatment with each of these metabolic inhibitors at late blastula caused an about 2-fold increase in the intensity of the three AU-binding proteins (densitometer tracing data is not shown).

4. DISCUSSION

The observation that certain mRNAs containing a 3′ AU-rich sequence, which generally turnover very rapidly, appear to be very stable in *Xenopus* oocytes suggested that these unique cells may lack some *trans*-acting factors that are required for the rapid turnover of these mRNAs [2]. We have previously shown that human SV80 cells, which naturally produce hu-IFNβ, contain three major proteins that specifically bind to the 3′ AU-rich region of hu-IFNβ mRNA [14]. In this work, we showed that similar proteins are also present in *Xenopus*. This similarity indicates that AU-binding proteins are evolutionary conserved and supports previous observations showing high conservation of RNA–protein complexes from frog to man [22].

Notably, only two of these AU-binding proteins (AU-F1 and AU-F3) were detected at relatively low amounts in *Xenopus* oocytes and eggs where mRNAs containing 3′ AU-rich region are unusually stable. Interestingly, following fertilization, when endogenous AU-rich mRNAs become unstable in *Xenopus*, the levels of AU-F1 and AU-F3 were elevated and AU-F2 was first detected in the gel. We can not yet discriminate whether AU-F2 is expressed only after fertilization or it is present in very low levels also in oocytes and eggs.

Since new transcription in *Xenopus* embryos is detected only after the 12th cell division, at the midblastula transition [23], the increased binding activity of the three factors before midblastula is apparently regulated post-transcriptionally. This was indeed confirmed by our results showing that the level of these proteins was reduced upon treatment of the embryos with cyclohexamide, but not actinomycin D before midblastula. Interestingly, treatment with each of these metabolic inhibitors at late blastula, when new transcription has already been established [23], caused a considerable increase in the intensity of all three proteins. Although the significance of this observation is yet unknown, it suggests that at late blastula there is active expression of genes whose protein products influence either the stability or the binding activity of these AU-binding factors. A similar observation was also previously reported showing that cyclohexamide increased the accumulation of two cytoplasmic factors in LpsnPM cells that are capable of interaction with the 3′ AU-rich sequence of hu-IFNβ mRNA [24].

At the present, we can not be certain of the role of AU-F1, AU-F2 and AU-F3 in the translation and instability of AU-rich mRNAs. However, based on the correlation between the induction of these AU binding factors and the developmental regulation of mRNA destabilization, caused by the 3′ AU rich region [4–7,16,25], it is tempting to suggest that AU-F2, or all three AU- binding proteins are involved either directly or indirectly in this destabilization process.

The mechanism of decay of the AU-rich mRNAs is

also unknown. Previous studies have shown that the AU-rich motif by itself is not necessary for mRNA degradation and that other regions located within the coding regions were found capable of conferring mRNA instability [26–28]. The degradation of certain mRNAs, including that of IFNβ, was found to be coupled to their ongoing translation [29–31]. Hence, the turnover of AU-rich mRNAs may also be indirectly influenced by factors that affect their translational activity. This hypothesis may be supported by several lines of evidence: (i) we have previously shown that the AU-rich 'destabilizing' region of hu-IFNβ may inhibit its translation by iteracting with the 3′ poly(A) tail, suggesting a possible mechanism for concomitant activation of both translation and destabilization of this mRNA by shortening of the poly(A) tail [14], (ii) in yeast, shortening of the poly(A) tail was suggested as a potential maturation step of mRNAs making them competent for efficient translation [32], and (iii) shortening of the poly (A) tail of c-*fos* mRNA was shown to be mediated by the 3′ AU-rich region [29]. Considering these observations, we can not exclude the possibility that the AU-specific binding proteins are involved in translational regulation of AU-rich mRNAs and by that indirectly affect mRNA turnover rate. Additional experiments are needed to unravel this important regulatory mechanism.

Acknowledgments: We are thankful to Dr. S. Pestka (Rutgers, NJ, USA), for providing recombinant interferon clones and to Dr. J. Chebath (Weizmann Institute of Science, Rehovot, Israel) for providing SV80 cells. G. Galili is the incumbent of the Abraham and Jenny Fialkow Career Development Chair in Biology.

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